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Mycobacterium tuberculosis isolates from Rio de Janeiro reveal unusually low correlation between pyrazinamide resistance and mutations in the *pncA* gene

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ABSTRACT

It has been widely accepted, that pyrazinamide (PZA) resistance in *Mycobacterium tuberculosis* is correlated with mutations in the *pncA* gene. But since years researchers have been puzzled by the fact that up to 30% of PZA resistant strains do not show any correlation between PZA resistance and mutations in the *pncA* gene, and thus may vary with geographic area. The objective of the study was to investigate the correlation between PZA susceptibility and mutations in *pncA* gene in *M. tuberculosis* isolates from individuals living in a highly endemic area. Therefore we analyzed drug resistant and multidrug resistant (MDR) isolates from patients in Rio de Janeiro, Brazil. From a total of 97 clinical isolates of *M. tuberculosis* 35 were identified as PZA resistant, 24/35 strains did not show PZase activity and 15/24 (62.5%) strains possess mutation in the *pncA* gene. This is a low correlation between PZA resistance and PZase activity (68.6%) and even lower correlation between PZA resistance and the presence of mutation in *pncA* gene (45.7%). Most of the mutations found were conserved near the active site or metal binding site of PZase. The 146A > C mutation was found both in PZA resistant and susceptible isolates, suggesting that this mutation may not fully associated with PZA resistance. Of the mutations found, three have not been previously described. The insertions 192–193 TCCTCGTC and 388–389 AGGTCGATG, although found before, here was found to be a short tandem repeat and in one strain, insertion of the IS6110 was observed 55 nt upstream of the gene. All PZA resistant isolates had no mutation in the gene coding ribosomal protein S1 (*rpsA*), which has recently been proposed as alternate target for pyrazinoic acid (POA). The results show a low association of PZA resistance and *pncA* gene mutations in a selected patient group from an highly endemic area. Our findings point out that the phenotypic susceptibility testing remains important for the detection of PZA-resistant *M. tuberculosis*.

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1. Introduction

Tuberculosis (TB) still remains one of the leading causes of morbidity and mortality in the world. At present one-third of the world's population is infected with *Mycobacterium tuberculosis*, the causative agent of TB. In 2011, 1 million people HIV negative and four-hundred and thirty thousand HIV positive died from the disease, 8.7 million new cases are estimated, of which 13% are coinfecting with HIV (WHO, 2012).

Pyrazinamide (PZA) is a first-line oral agent for TB short course chemotherapy. It is one of the few anti-TB drugs effective against persistent tubercle bacilli. It is only active against *M. tuberculosis* and *Mycobacterium africanum* in the host, while *Mycobacterium bovis* is inherently resistant to it. Since the global resurgence of drug resistant TB strains, an increasing number of PZA resistant strains have been observed. PZA is a prodrug which is converted into the active substance pyrazinoic acid (POA) by *M. tuberculosis* at low pH medium (Hewlett et al., 1995; Mc Dermott and Tompsett, 1954; Tarshis and Weed, 1953).

In *M. tuberculosis* PZA is converted to POA by a 19.6 kDa pyrazinamidase (Rv2043c), encoded by *pncA*, a gene which spans 561 base pairs (Blanchard, 1996). PZA susceptible *M. tuberculosis* possesses a functional PZase, and mutations in this gene have been reported leading to reduction or complete loss of PZase

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activity, which in turn make the bacteria resistant to PZA (Butler and Kilburn, 1983; McClatchy et al., 1981). Despite a majority (70–97%) of PZA-resistant isolates of *M. tuberculosis* exhibit mutations in their *pncA* gene or putative regulatory region, PZA resistant isolates with PZase activity and wild type *pncA* sequences have also been reported in several studies, indicating the existence of alternate molecular mechanism of PZA-resistance (Ngo et al., 2007; Raynaud et al., 1999; Zhang and Mitchison, 2003). Recently the 30S ribosomal protein S1 (*rpsA*) was found to bind to POA and subsequently inhibiting trans-translation. Mutations in the *rpsA* gene were associated with PZA resistance in clinical isolates without mutations in *pncA* (Shi et al., 2011). We studied the relation between PZA resistance, PZase activity and mutations in *pncA* and or *rpsA* in clinical strains isolated from TB patients in a highly endemic area of Brazil, Rio de Janeiro state (57.6/100,000 inhab).

2. Materials and methods

2.1. Clinical strains and identification

A total of 97 *M. tuberculosis* clinical strains were exclusively isolated from sputum samples collected from patients who were diagnosed with susceptible or resistant TB attended at University Hospital Clementino Fraga Filho, and Hospital Municipal Raphael de Paula Souza, Rio de Janeiro, Brazil between 2003 and 2005. University Hospital Clementino Fraga Filho is a tertiary care hospital that attends patients with TB and co-morbidities including seropositive HIV and has no spontaneous demand. Hospital Municipal Raphael de Paula Freitas is a reference center for MDR-TB cases. The clinical specimens were treated with N-acetyl-L-cysteine-NaOH and 0.2 ml of the pellets was inoculated in tubes with Löwenstein–Jensen (LJ) medium. The genus *Mycobacterium* was confirmed by Ziehl–Neelsen staining and as *M. tuberculosis* complex by biochemical tests such as: nitrate reduction, niacin production and catalase activity ().

2.2. Drug susceptibility testing and PZase activity assay

The *M. tuberculosis* isolates were tested for PZA susceptibility by proportion method in LJ medium pH 5.5–5.8 with PZA at 100 µg/ml (Singh et al., 2007). In addition the isolates were tested for streptomycin (STM), isoniazid (INH), ethambutol (EMB) and rifampicin (RPM) susceptibility using the standard proportion method on LJ medium. An isolate was defined as resistant when the percentage of colonies exceeded 1% of the growth on a drug-free medium (control) at the critical concentrations of 4, 0.2, 2, and 40 µg/mL, respectively (Canetti et al., 1969). Multidrug resistance (MDR) is defined as resistance to at least INH and RMP, resistance to two or more drugs but not to MDR was assigned as drug resistance, and resistance to any one drug as monoresistant. PZase activity was determined using a modified method described by Wayne (Wayne, 1974). In brief, a heavy loopful of mycobacterial culture freshly grown on LJ medium was inoculated in two 16 × 125 mm glass tubes with screw cap containing 5 ml Dubos broth medium supplemented with 100 µg/ml PZA and 2 mg/ml sodium pyruvate. After incubation at 37 °C for 4 or 7 days (when negative at the fourth day the second tube was incubated for three more days), 1 ml of freshly prepared 1% ferrous ammonium sulphate solution was added to each tube, and the presence of a pink band was assessed. *M. tuberculosis* H37Rv and *Mycobacterium fortuitum* were used as a positive and negative control, respectively for the assay. Strains giving discordant results in phenotypic and genotypic analysis were retested for PZA susceptibility (proportion method and

BACTEC Mycobacteria Growth Indicator Tube (MGIT), as recommended by Bencton Dickinson) and PZase activity.

2.3. DNA isolation

Chromosomal DNA was isolated as described by van Embden et al. (1993) from a loopful of clearly visible mycobacterial fresh growth in LJ. The purified DNA was dissolved in 20 µl of 0.1 × TE buffer and stored at –20 °C until used. DNA concentration was defined using a spectrophotometer Nanodrop 1000 (ThermoScientific, USA).

2.4. PCR and sequencing

The gene *pncA* was amplified with the pair of primer *pncA*-F and *pncA*-R which produced 772 nt fragment. This included 81 nt upstream of *pncA* and 130 nt downstream the gene. The PCR was done in a MJ Research PTC-200 thermo cycler (Harlow Scientific, Arlington, MA) with 10 µM of forward and reverse PCR primers, 200 µM dNTP, 1.5 mM MgCl₂, 1X Qiagen PCR Buffer and 0.5 U Taq DNA Polymerase (Qiagen Master Mix, Qiagen, Germany) using 0.02–0.04 µg of genomic DNA in a total volume of 20 µl. PCR conditions used was an initial denaturation at 95 °C for 10 min; followed by 30 cycles of sequentially denaturation at 95 °C for 30 s, annealing at 57 °C for 30 s and polymerization at 72 °C for 50 s. The final annealing was done at 95 °C for 10 min. Sequencing of *pncA* was done with the primers *pncA*-F, *pncA*-R, *pncA*-sf1, *pncA*-sf2, *pncA*-sr1, *pncA*-sr2. For the detailed analysis of the *pncA* promoter region and additional 454 nucleotides upstream *pncA*, the primers Rv2044cF and Rv2044cR were used for amplification and sequencing. The PCR was carried out as described for *pncA* except 30 s were used for the extension step. The IS element of strain number 13 was analyzed with the primers Transp-sf and Transp-sr. All primers which were used for amplification and sequencing of *pncA* are summarized in Table 1 and Fig. 1.

Sequencing of *rpsA* was done with the primers *rpsA*-F and *rpsA*-R (Table 1). The 1600 nt PCR product, included 130 nt upstream of the gene. *RpsA* was amplified with BioMix (Bioline GmbH, Germany). The PCR was performed as described for *pncA* except 100 s were used for elongation. The PCR products were purified with a QIAquick PCR purification kit (Qiagen GmbH, Hilden, Germany).

Table 1
Primers for *pncA* and *rpsA* used in the study.

Name	Primer sequence 5' → 3'	Description
<i>pncA</i> -F	CGGCGTCATGGACCTATA	Amplification and sequencing of <i>pncA</i>
<i>pncA</i> -R	GAACCCACCGGTCTTCG	Amplification and sequencing of <i>pncA</i>
<i>pncA</i> -sf1	CATTGCGTCAGCCGTACT	Sequencing of <i>pncA</i>
<i>pncA</i> -sf2	CCACCGATCATTGTGTGC	Sequencing of <i>pncA</i>
<i>pncA</i> -sr1	GCAGCCAATTCAGCAGTG	Sequencing of <i>pncA</i>
<i>pncA</i> -sr2	GGTCGATGTGGAAGTCCT	Sequencing of <i>pncA</i>
Rv2044cF	TGGCGAATTGAACCTTTCT	Amplification and sequencing of 454 nt upstream of <i>pncA</i>
Rv2044cR	CACCCTCGCAGAAGTCGT	Amplification and sequencing of 454 nt upstream of <i>pncA</i>
Transp-sf	ACCGAAGAATCCGCTGAG	Sequencing of IS6110
Transp-sr	TCGCGTCGAGGACCATGGA	Sequencing of IS6110
<i>rpsA</i> -F	CGATTTTCCGCCCTGAGT	Amplification and sequencing of <i>rpsA</i>
<i>rpsA</i> -R	ACGTGAACGCGATCAGCTG	Amplification and sequencing of <i>rpsA</i>
<i>rpsA</i> -sf1	ACATCGGGCTGCGGGTTTCC	Sequencing of <i>rpsA</i>
<i>rpsA</i> -sf2	AGCGTCACGTCGAGGTGC	Sequencing of <i>rpsA</i>
<i>rpsA</i> -sr1	ACTTGGTGACCTTGCCCC	Sequencing of <i>rpsA</i>
<i>rpsA</i> -sr2	TTGAGCGCCTCGATGGTGC	Sequencing of <i>rpsA</i>

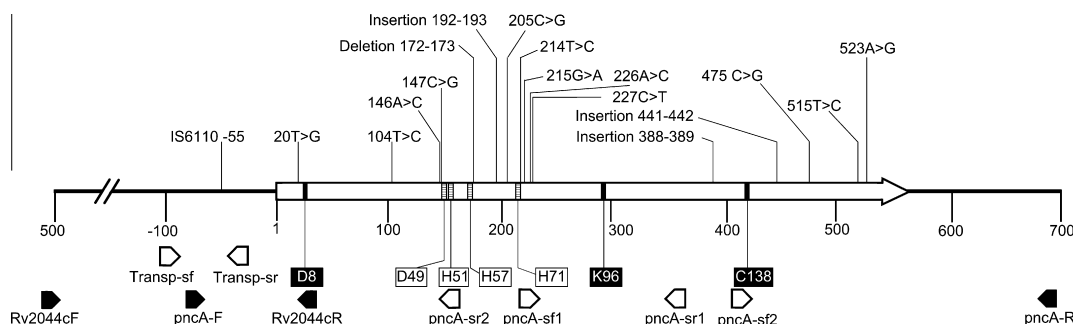


Fig. 1. Depiction of the *pncA* gene and the position of the identified point mutations and insertions in this study. Sequencing primers are shown as short white arrows, PCR primers as short black arrows. The positions of triplets, coding for the residues of the metal binding site and substrate binding site are shown as striped and black boxes, respectively.

The sequencing of the extracted DNA was performed with an Automated 3730xl DNA Analyzer (Applied Biosystems, Inc, USA) using the same PCR primer set and sequencing primers (Table 1). The DNA sequences were analyzed by comparison with reference strain *M. tuberculosis* H37Rv DNA sequence using Pregap version 1.6 and Gap4 version 4.11 of Staden Package (Staden et al., 2000). Mutations comparison was performed by searching in the TB Drug Resistance Mutation Data Base (Sandgren et al., 2009; http://www.tbdreamdb.com/PZA_Rv2043c_HighConfidenceMutations.html).

3. Results

3.1. Characteristics of isolates, PZA-susceptibility, and mutations in the *pncA* gene

A total of 97 *M. tuberculosis* clinical isolates were analyzed for PZA-susceptibility, PZase activity and mutation in *pncA* gene. Sixty-two isolates were pyrazinamide susceptible and 35 were pyrazinamide-resistant. Twenty-two of the 35 pyrazinamide-resistant (PZA^R) strains were MDR (63%). From the 13 non-MDR strains were four strains PZA mono-resistant. In addition to the PZA resistance were two strains resistant to INH. Three strains were resistant to INH and STM. There were three single strains resistant to EMB + RMP, STM + EMB, and INH + EMB. Sixty-two isolates were pyrazinamide susceptible (PZA^S), of which 38 were pan susceptible (61.3%) to all four assayed drugs and 10 were mono-resistant to any one of the four drugs (16.1%) and seven were MDR (11.3%). Seventeen PZA^S strains (25.8%) were resistant to STM and EMB in different combinations.

In 24 out of 35 PZA^R isolates (68.6%) we could not detect PZase activity. This is a low correlation related to other studies, however similar (65.4%) found in Thailand (Jonmalung, 2010). Sixteen of these 35 PZA^R strains possessed mutation in the *pncA* gene (45.7%), and 62.5% of PZase-negative strains possessed a mutation in the *pncA* gene, however concordance for all tests was 42.8% (Table 2).

3.2. Distribution of mutations in the *pncA* gene

Among the studied strains the mutations in *pncA* were distributed throughout the gene as in previous studies (Barco et al., 2006; Lemaitre et al., 1999; Raynaud et al., 1999; Singh et al., 2007; Somoskovi et al., 2007). Point mutations were observed in 14 strains, causing amino acid substitutions, which are located near the active site or metal binding site (active site: V7; metal site: D49, C72, T76) (Lemaitre et al., 1999; McClatchy et al., 1981; Scarpio et al., 1997; Somoskovi et al., 2007; Zhang and Mitchison, 2003). Other isolates bearing different mutations at position 49 showed PZA-susceptibility as well as PZA-resistance. For example, isolates identified by the numbers n# 25, 37 and 47 have a D49A mutation in the *pncA* gene but show a contrasting phenotype concerning PZA susceptibility (Table 3). Such contrasting results for strains bearing a mutation near the active site were also encountered in previous studies (Dormandy et al., 2007; Hirano et al., 1997; Somoskovi et al., 2007; Sreevatsan et al., 1997). Structure of PZase recently published showed the aspartic acid at position 49 binds directly to the metal in the enzyme (Petrella et al., 2011). A summary of all observed mutations is shown in Fig. 1. None of the analyzed strains had mutation in putative promoter region of *pncA*. Even up to 454 nt upstream of *pncA* mutation was not observed (data not shown).

3.3. Insertions and analysis of IS6110

In five strains 8, 2 and 9 nt insertions (TCCTCGTC, CG, AGGTCGATG) were observed between position 192–193, 444–445 and 388–389, respectively. The 8–9 nt inserts TCCTCGTC and AGGTCGATG produced a short tandem repeat of (AGGTCGATG)₂ and (TCCTCGTC)₂ in *pncA* gene, respectively (Fig. 1). The 9 nt insertion in codon 130 led to deletion of V130 and insertion of additional three amino acids. Though the insertion AGGTCGATG between positions 388–389 was previously reported (Sreevatsan et al., 1997) it was not known to produce tandem repeat as is being reported now. These repeats are very likely remains of a mobile ele-

Table 2

Summary of pyrazinamide (PZA) susceptibility, PZase activity and changes in *pncA* observed in the clinical *M. tuberculosis* isolates.

Total Strains: 97				PZA resistant strains: 35			
PZA susceptible strains: 62							
PZase + : 50		PZase – : 12		PZase +: 11		PZase – : 24	
Wild type	Mutant	Wild type	Mutant	Wild type	Mutant	Wild type	Mutant
46	4	12	0	10	1	9	15

PZase +/-: pyrazinamidase positive/negative.

Table 3
pncA gene mutations detected in clinical *M. tuberculosis* isolates.

Strain No	PZA susceptibility [#]	PZase activity [§]	Amino acid change	Mutation
<i>Point mutation</i>				
103	R	–	V 7 G	20 T > G
92	R	–	L 35 P	104 T > G
37	S	+	D 49 A	146 A > C
47	R	–	D 49 A	146 A > C
25	R	–	D 49 A	146 A > C
13/04	S	+	D 49 E	147 C > G
36	R	–	P 69 A	205 C > G
82	R	–	C 72 R	214 T > C
41	R	–	C 72 Y	215 G > A
107	R	–	T 76 P	226 A > C
4	R	–	T 76 I	227 C > T
20	R	–	L 158 P	457 C > G [*]
23	R	–	L 172 P	515 T > C
66	R	–	M 174 V	523 A > G
<i>Insertion</i>				
26	R	–	Frameshift	192–193 TCCTCGTC [*]
101	R	–	Frameshift	192–193 TCCTCGTC [*]
32	R	–	Frameshift	192–193 TCCTCGTC [*]
89	R	–	V 130EVD	388–389 AGGTCGATG
40	R	–	Frameshift	444–455 CG [*]
<i>Deletion</i>				
33	R	–		172–173 Deletion 2 nt [*]
<i>Insertion Elements</i>				
13	S	+		IS6110 at –55 nt [*]

PZA: pyrazinamide. PZase +/–: pyrazinamidase positive/negative.

[#] R: resistant, S: susceptible.

[§] +: active, –: inactive.

^{*} Unreported mutation, insertion, or deletion.

ment. This is supported by further finding of a strain comprising IS6110 located 55 nt upstream of the *pncA* gene (this study) and presence of disrupting *pncA* (Lemaitre et al., 1999).

3.4. Mutational analysis of the *rpsA* gene

None of the PZA-resistant strains harbored mutations in the *rpsA* gene.

4. Discussion

PZA resistance has been correlated with mutations in *pncA* gene, which codes for a pyrazinamidase, converting the prodrug PZA into POA. Correlation of drug resistance with a defect in a specific “drug resistant” gene is observed for several drugs. Resistance against ofloxacin, rifampicin, ethambutol, and isoniazid show variable correlation between the drug resistance phenotype and mutations in the drug resistance genes *gyrA* (75–94%), *rpoB* (90–95%), *embB* (47–65%), and *katG* (31–97%) /*inhA* (8–43%), respectively (TCCTCGTC) (Zhang and Yew, 2009).

In several studies the correlation of PZA resistance and mutation in *pncA* has also been reported in a variety of low range (41–80%) (Bishop et al., 2001; Huang et al., 2003; Miyagi et al., 2004; Rodrigues et al., 2005; Chan et al., 2007; Pandey et al., 2009; Jonmalung et al., 2010). While in other the correlation has been reported higher such in South Africa (91–92%) (Louw et al., 2006; Mphahlele et al., 2008), China (91%) (Hou et al., 2000), Japan (97%) (Hirano et al., 1997), S. Korea (97%) (Lee et al., 2001). In the studied strains, only a correlation of 45.7% between PZA resistance and *pncA* mutation was observed which is far lower than the majority studies performed so far, including reports from low endemic area in Brazil (72% and 84%) (Rodrigues et al., 2005; Barco et al., 2006), although similarly found in South Africa (67%) and Taiwan (41%) (Bishop et al., 2001; Huang et al., 2003). Such a lower

correlation might be attributed to some extent due to the limitation of phenotypic susceptibility test based on LJ proportion which was done at pH 5.5–5.8 with 100 µg/ml PZA. False resistance is the most common cause of error in the results of susceptibility testing to PZA, and recently Simons et al. (2012) reported that this occurs under the influence of inoculum. The ammonia produced by bacterial metabolism lead to increases the pH of the culture medium and hence inactivation of the drug (Mc Dermott and Tompsett, 1954; Zhang et al., 2002). However susceptibility testing was repeated carefully, including using MGIT for same discordant strains. The limitation of the test is not the sole reason for contribution of low correlation between PZA resistance and *pncA* mutation is supported by the presence of strains which were resistant depending on concentration of PZA used (Dormandy et al., 2007).

On the other hand, it is important to note that most of these strains are drug resistant or MDR and according to several authors resistance to PZA is associated with strains harboring multiple resistance to the first line drugs (>49%), (Chang et al., 2011; Chiu et al., 2011; Fonseca et al., 2012; Kim et al., 2012; Louw et al., 2006; Minime-Lingoupou et al., 2010; Mphahlele et al., 2008; Shenai et al., 2009). Frequency in *pncA* mutation among MDR strains was similar found in S. Korea (56.2 × 63.9%). So, it must have a reasonable explanation of the presence of these complex patterns which are most probably not a result of a defect in a single gene. The mutation found was diverse and scattered along the gene. Mutation is not always associated with resistance as described here and by other Jonmalung et al. (2010).

As the PZA susceptibility and *pncA* mutation were not highly correlated in the current studies, we also sequenced *rpsA*, which codes for the ribosomal protein S1, which has been reported as additional target for PZA resistance (Shi et al., 2011). None of the resistant strains had a mutation in the *rpsA* gene. Recent study on analyze of PZA^R strains reported *rpsA* gene has no impact on PZA resistance, although authors have found few mutations could

not clearly associate with strains phenotypes (Alexander et al., 2012).

The results show a low association of PZA resistance with mutations in the *pncA* gene in isolates from patients living in a highly endemic area. The results jeopardize the use of methods which use *pncA* as sole indicator for the detection of PZA resistance. These informations may be helpful in the TB clinical practice for the selection and interpretation of diagnostic tests.

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